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The healthy effects of L. Barbarum on "in vitro" cell models

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ABSTRACT

Cancer is a group of diseases characterized by the over proliferation of the cells. Epidemiological studies indicate that about 30-40% types of cancers are directly or indirectly linked to improper diet and related factors. Morover, several reports demonstrate an association between intake of fruits and vegetables and reduced mortality from degenerative diseases, including cancer.
Plants possess a variety of bioactive substances like phenols, flavonoids, carotenes and organo sulphur compounds having anti proliferative activities.
Goji is a native Chinese deciduous shrub with bright red berries. Goji has been utilized in traditional chinese medicine since the first century AD to promote longevity, to sustain the liver and improve the eyesight.
To date, several studies indicate beneficial effects of Goji berries for diabetes, high blood pressure, poor circulation, fever, malaria, inlammatory diseases, eye disorders and cancer.
Goji polysaccharide was shown to inhibit the growth of human leukemia HL-60 and human breast cancer MCF-7 cell lines. In vivo, it could enhance the anti-cancer effect of a chemotherapy on patients with various neoplasias, such as malignant melanoma, renal cell carcinoma, colorectal carcinoma, and lung cancer.
Besides the great variety of association studies, there is a need of studies to unravel its therapeutic effects at the biochemical level.
In our study, we demonstrate that Goji dry extracts do not induce in vitro tumor cell death and do not determine reduction of proliferation, using several tumor cell lines and fibroblasts and lymphocytes as normal cell controls.
Goji dry extract has shown in vitro excellent anti-inflammatory properties in a bidimensional monolayer of

differentiated Caco-2 cells, as a model of the intestinal barrier. TNF-alpha-induced increase in intestinal epithelial tight junction (TJ) permeability, proposed as one of the proinflammatory mechanisms contributing to the intestinal inflammation, has been drastically reduced by “Goji” treatment; moreover, after 24 h incubation with the cytokine, MTT showed a beneficial protective effect when epithelial cells were treated with the Goji extract.
In our study, we have also evaluated the cellular effects by the fecal water (aqueous phase of human feces, FW), a useful biomarker approach to study cancer risks and protective activities of food.
Faecal waters from 5 healthy volunteers consuming their habitual diet were screened for genotoxicity by the single-cell gel electrophoresis (comet) assay and citotoxicity by the MTT assay, using a human cell line models, as target; goji berries supplementation increased the vitality of FW-treated cells and reduced genotoxic damages, confirming the antioxidative properities of goji.
The results showed remarkable biological activity of goji berries and dry extract and qualify it as a potential resource for food/pharmaceutical applications.

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1. INTRODUCTION

Recently there is a growing interest on the microflora as a factor implicated in the development of metabolic, neoplastic and inflammatory diseases.

Understanding the microbiota functions involves a change of perspective: our health is programmed not only through DNA sequences, but also depend on the epigenetic changes that the microbiota carries the expression of our genes.

The gut microbiota is a complex ecosystem characterized by a dynamic and reciprocal interaction with the host.

Thanks to this interaction, the microbiota is able to perform critical functions to the health of the host, such as digestion of complex carbohydrates of vegetable origin, vitamins and short-chain fatty acid production, interaction with the immune system, regulation of some extracellular signaling pathways and competing with pathogenic or opportunists bacteria.

The composition of the intestinal microbial ecosystem and the balance of its interaction with the host are increasingly seen as a key factor not only for the health of the gastrointestinal tract, but the whole organism (Conlon MA and Bird AR, 2015).

The human gastrointestinal tract is inhabited by microorganisms, which are more than 10 times the number of human cells in our bodies (Burcelis R et al., 2013). They carry out different functions about fermentation of residual food, modulation of gut immune function, and protection against pathogens and diseases. Although

the intestinal microbiota is largely beneficial, changes in bacterial populations or in the products of bacterial metabolism may contribute to disease (Zoetendal EG et al., 2008; Ley RE, 2010).

In view of these considerations, humans can be considered a superorganism composed of microbial and human cells, whose genetic heritage is represented by human genes and genome of the intestinal microbiota (microbiome) (Gill et al., 2006).

The intestinal microbiota develops after birth, its characteristics depend on the different microorganisms species whereby they come into contact in the early years of life and the genetic individual. At birth the intestine is essentially sterile, the first year of life is critical to the development of the microbiota, which increases between the first and the fourth year of age, then it evolves into adulthood and remains stable over time; its composition is different for each individual due to different bacterial species (Torroni F et al., 2009; Palmer C et al., 2007; Gronlund MM et al., 1999; Armsen HJ et al., 2000).

Approximately 1000 species of different bacteria both aerobic and anaerobic were identified; the concentration and the composition of the microbial population depend on various environmental factors present in the different districts of the intestinal tract, in addition, the diet may also influence the composition of the microbial community resident. The microbial species in the gut may be transient or permanent; the majority of them are commensals, some are potentially pathogenic, others play a beneficial role in the health of the host. The protective role of the symbiotic bacteria is important because they constitute an efficient barrier against colonization of enteropathogenic microorganisms, as well as their interaction with the host immune

system, a critical element for the establishment of an immune tolerance that allows the maintenance of 'homeostasis.

Regarding the composition of the microbiota, it is necessary to consider that the digestive system does not constitute a homogeneous environment, as it is characterized by a significant increase of the pH in the lumen and for a progressive decrease in the amount of oxygen progressing from the stomach to the colon.

The microbiota is considered a real organ that carries out some functions that our body is not able to carry out, such as the digestion of indigestible components present in our diet such as polysaccharides contained in plant foods (Rafter JJ et al. 1987).

Before the foods are metabolized by intestinal bacteria, the metabolic activity of the microbiota can alter the exposure of the host to these components and their potential health effects (Conlon MA and. Bird AR, 2015).

Therefore the microbiota is composed mainly of lactobacilli and streptococci in the stomach and duodenum, where stomach acid, bile produced by the liver and pancreatic secretions inhibit the germination and colonization of microbes ingested with food, while the intestinal microenvironment and especially the colon is the ideal habitat for the survival and growth of bacteria, particularly anaerobic bacteria.

In the colon where the amount of bacteria and the composition of the microbiota change drastically, you can find concentrations of microorganisms of up to 10^{12} CFU/ml, with prevailing presence of Bacteroides, Bifidobacterium, Lactobacilli (Eckburg PB et al., 2005).

2. COMPOSITION OF MICROBIOTA

The gut microbiota contains all three domains of life, bacteria, archeobacteria and eukaryotes. The majority of the bacteria comprising the microbiota are uncultivable, which meant in the past that researchers were unable to characterize them. The new genetic technologies to study the bacterial flora have allowed researches to identify and study the "metabolome", the whole superorganism composed of the microbiota and host genome, and its influence on health and disease.

Next generation sequencing and molecular taxonomic technologies are filling the gaps left by conventional microbiology techniques to better understanding the normal microbiota.

The phylogenetic analysis of bacterial 16S rRNA genes provides an efficient strategy for exploring the biodiversity of microbiota (Azcárate-Peril MA et al., 2011). In human fecal samples different taxa were identified and wide majority (95%) of them distributed among three major bacterial group: the *Bacteroides* spp, the *Clostridium coccoides* spp and the *Clostridium leptum* spp. The predominant genera in the large bowel are reported to be Bacteroides, Eubacterium, Clostridium, Ruminococcus, Peptococcus, Peptostreptococcus, Bifidobacterium, and Fusobacterium (Suau A et al., 1999) (Fig. 1).

However, this concept is being continuously redefined as more data are collected.

The recent data generated by deep sequencing of the human intestinal microbiota has revealed that most bacterial species are present at low abundance (species defined as organisms sharing

>97% sequence identity in their 16S rRNA genes)(Qin J et al 2010).

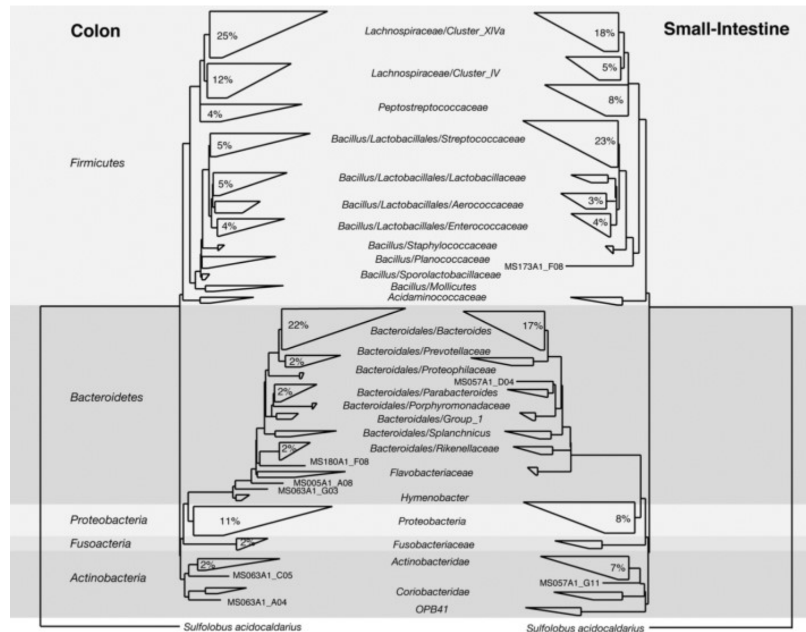


Fig.1 Comparison of SSU rRNA sequences isolated from colon and small intestine samples

The intestinal microbiota differs in terms of quality along the gastrointestinal tract, in addition to these differences there is a horizontal stratification, with the presence of various microbial communities in the intestinal lumen and in the layer of mucus in the crypts (Rozee et al., 1982).

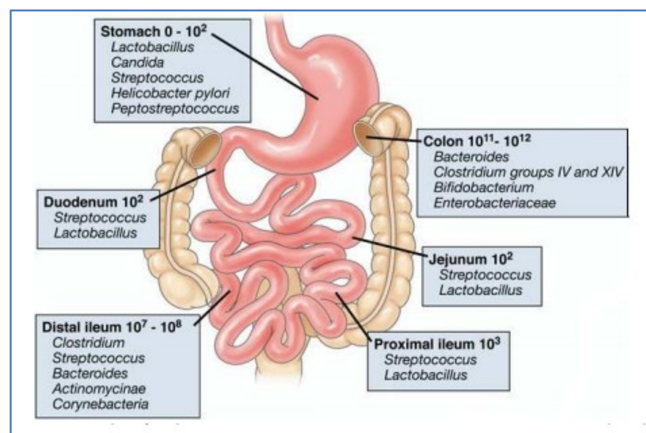


Fig.2 Composition and concentrations of luminal microbial species dominant in gastrointestinal tract

Quantitatively, esophagus and stomach carry the lower bacterial load; the normal esophageal microbiota is relatively simple in composition, facultative anaerobes are present that derived from the oral cavity, such as Streptococci and Lactobacilli. The stomach contains low levels of lactobacilli and other acid resistant microorganisms, generally coming from the oral cavity. The stomach is a barrier against the access of bacteria from the external environment towards the distal parts of the gastrointestinal tract due to factors such as acidity, the presence of nitric oxide which derives from bacterial reduction of salivary nitrate and proteolytic enzymes.

Progressively, the concentration of bacteria increases along the intestinal tract due to the decrease in the redox potential. The large intestine is the site of the gastrointestinal tract with the higher number and greater diversity of bacteria (Fig. 2).

Therefore in the human intestine there is a microbial diversity that is the result of co-evolution of microbial communities and their guests. This diversity derives from a process of natural selection operating at the host level and it favors the initiation of stable communities with a high degree of functional redundancy (Yachi and Loreau, 1999).

The functional redundancy avoids the onset of species with a central role in ecosystem and this loss would lead to a dramatic change in terms of processes and diversity. The opposite force makes a selective pressure arising from the competition between members of the microbiota and it promotes their functional improvement.

Among the factors that influence the microbial diversity an examples is given from the chemical environment that plays an

important role, in addition also the intestinal peristalsis that limits the number of microbial niches available (Ley et al., 2006).

3. FUNCTIONS OF THE MICROBIOTA

The intestinal flora plays essential functions in health care, because it has a metabolic, structural and protective roles.

Molecular analysis has shown that commensal bacteria modulate the expression of genes involved in several intestinal and extra intestinal functions, including xenobiotic metabolism, postnatal intestinal maturation, absorption of nutrients and improvement of the mucosal barrier.

The main metabolic function of the microbiota is represented by fermentation of the non-digestible diet residues and mucus endogenous produced by epithelium, which are major source of energy in the colon; the fermentation of residues provokes the metabolic production of short-chain fatty acid (SCFA).

In the cecum and right colon fermentation is very intense with a high production of SCFA, due to an acid pH (range 5-6) and a rapid bacterial growth.

By contrast, in the distal colon the substrate is less available, the pH is neutral, the putrefaction process increased and bacterial activity is lower.

The putrefaction is the anaerobic metabolism of peptides and proteins (collagen and elastin from food residues, enzymes pancreatic, exfoliated epithelial cells and bacteria lysates, etc.) that also produces SCFA but, at the same time, it generates potentially toxic substances including: ammonia, amines, phenols, thiols and indoles (Macfarlane GT et al.,1986).

The SCFA have important functions in host physiology:

1) butyrate is the main source of energy for the epithelium of the colon;

2) acetate and propionate are metabolized by the liver (propionate) or peripheral tissues, particularly the muscles (for acetate), and they may have a role as modulators of glucose and cholesterol metabolism (Wong JM et al.,2006; Chen W et al., 1984; Berggren AM et al., 1996).

The synthesis of fatty acids causes acidification of the intestinal pH and it is an efficient system of defense against pathogens. In addition the production of SCFA allows the growth of intestinal epithelial cells, promoting their proliferation and differentiation. (Guarner et al. 2006)

The bacteria that produce SCFA appear to influence the cycle of enterocytes in the colon; especially butyrate inhibits cell proliferation, stimulates differentiation in neoplastic epithelial cells in vitro and it appears to promote the returning from neoplastic phenotype in non-neoplastic (Lefebvre P et al.,2009).

The microorganisms of the colon also play a role in the synthesis of vitamins (B1, B2, B6, B12, PP, H, pantothenic acid and folic acid) and in the absorption of calcium, magnesium and iron; this role is further improved by the presence of SCFA (Bernet MF et al., 1994).

The intestinal microbiota has also other functions: detoxification of carcinogens, modulation of the host susceptibility to different cancer types, both inside and outside intestine and bile acids modification resulting in alterations of lipid metabolism of the host (Martin et al., 2007). On the basis of metabolic functions exercised by intestinal microflora, it is clear that the nutritional

food value is not, therefore, an absolute value but largely influenced by the digestive capacity of the individual microbiota (Bäckhed et al., 2004, 2007; Gill et al., 2006; Turnbaugh et al., 2006).

The trophic effect exerted on the intestinal epithelium by SCFA has a fundamental role in the physiology of the colon. All the 3 major SCFA (acetate, propionate and butyrate) stimulate in vivo the proliferation and differentiation of epithelial cells at the level of small and large intestines (Frankel et al., 1994).

The gut microbiota contributes to structural development of the gut mucosa by inducing the transcription factor angiogenin-3, which has been implicated in the development of intestinal micro-vasculature (Stappenbeck TS et al., 2002).

The resident bacteria are a fundamental line of resistance to colonization by exogenous microorganisms. They actively regulate the production of nutrients by a negative feedback mechanism, in order to prevent the availability of nutrients for potential pathogenic (Bernet MF et al., 1994; Hooper LV et al., 1999). In addition, through the production of antimicrobial substances, called bacteriocins, they compete for attachment sites in brush intestinal epithelial cells in order inhibit the growth of potential competitors pathogens (Brook J et al., 1999).

The intestinal mucosa represents the main surface of contact between the immune system and the external environment; at this level, the interaction between host and bacteria promotes development both the innate and adaptive immune systems. (Guarner and Malagelada, 2003).

In fact, Peyer's patches develop only after exposure to intestinal microbiota, and they are more abundant in the ileum, where the

number of bacteria is higher (Cebra JJ et al.,1998 ; Haverson K et al., 2007; Hapfeimeier S et al., 2010).

The mammalian immune system plays an essential role in maintaining homeostasis with resident microbial communities, thus ensuring that the mutualistic nature of the host-microbial relationship is maintained. At the same time, resident bacteria profoundly shape immunity (Hooper LV et al., 2012).

The components and the cell types from the immune system that participate in the immunomodulatory process includes the gut associated lymphoid tissues (GALT), effector and regulatory T cells, IgA producing B (plasma) cells, Group 3 innate lymphoid cells, and, resident macrophages and dendritic cells in the lamina propria (Fig. 3) (Jandhyala SM et al., 2015).

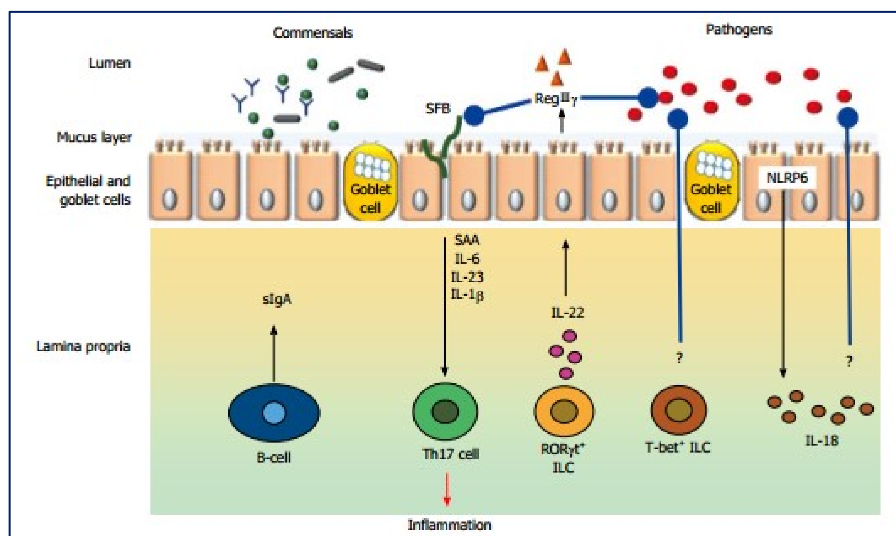


Fig. 3 Schematic representation of cell types and mediators involved in immunomodulation in the gut.

Certain bacteria and their products play a role in the development of tolerogenic anti-inflammatory T regulatory cells while others lead to the development of more proinflammatory Th17 cells as well as the programming of B lymphocytes to produce secretory

IgA that is secreted at mucosal surfaces. Further emphasizing this host microbial mutualism, the intestinal epithelium interacts with the gut microbiota through the production of nutrients in the form of mucus to support bacterial metabolism, as well as antimicrobial peptides that help to shape the structure of the gut microbiota (Wu GD and Lewis JD, 2013).

Therefore, the microbiota plays an important role on the development of the mucosal immune system, both in terms of physical components that function.

4. MICROBIOTA AND CANCER

The symbiotic relationship between human cells and microbiota is not well understood, further investigation are necessary. An increasing of knowledge suggests that the microbiota plays an important role in ''lifestyle-related illnesses''; identifying and distinguishing ''good'' from ''bad'' bacteria and how the microbiome may influence human health are considerable challenges. However, the influence of some bacteria and viruses on cancer development is well known (Hagland HR et al., 2013).

It has been estimated that approximately 20% of the human cancers worldwide are linked to microrganisms or infection-associated chronic inflammation, and examples are the induction of cervical cancer by human papillomaviruses and gastric cancer by Helicobacter pylori (Hausen HZ 2009; Warren JR, 2006).

Several evidences demonstrate that commensal microbiota - bacteria, fungi and viruses - exerts important effects on carcinogenesis, tumor progression and response to therapy. The effect of bacterial flora on cancer may be local and systemic, by the physiological communication between organism and microbiota through intact membrane or following alteration of barrier permeability in pathology (Dzutsev A. et al., 2015).

Genetic alterations are required for the proliferation of cancer cells within the tissue in which they originate, tumor progression and invasion are also dependent on the host response in terms of inflammation and antitumor immunity. This host response provides both a tumor-promoting environment and an immune barrier to tumor progression.

The presence of adaptive immune cells within the tumor has been shown to be an important predictor of progression and prognosis tumor (Angell GJ et al., 2013).

Mechanisms of microbial influence on cancer development in the intestinal tract include the balance of pro- and anti-inflammatory signals, the direct effects of bacterial enterotoxins on mucosal cells and intracellular pathways and the indirect potential of bacteria in the conversion of pro-carcinogenic dietary factors into carcinogens (Boleij A et al., 2012).

The intestinal microbiota may influence susceptibility to cancer through different contributions functional:

• harvesting inaccessible nutrients from the diet (i.e., fermentation of dietary fibers and resistant starch);

• metabolizing xenobiotics, including those that are potentially beneficial and detrimental (i.e., dietary constituents, drugs, carcinogens, etc.);

• renewing gut epithelial cells and maintaining mucosal integrity, affecting immune system development and activity (Smith PM et al 2013).

Scientists are now able to characterize the changes in the composition of the intestinal flora, known as dysbiosis, associated with several diseases through the next generation (Wu GD and Lewis JD., 2013).

Dysbiosis changes the immune regulatory systems that normally manage inflammation in the gut, and it is associated with immune-mediated disorders.

The changes in community function brought about by dysbiosis have also been investigated and compared using metagenomics analyses (Maynard CL et al., 2012).

A decrease of bacterial-dependent immune cell stimulation may be due to dysbiosis, resulting in a reduction in the overall number of bacteria and/or the abundance of specific species and encouraging a permissive environment for tumorigenesis (Xuan C, et al., 2014).

The epithelial barrier is maintained by active mechanisms mediated by soluble products (antibacterial peptides, antibodies), innate (macrophages, innate lymphoid cells, DC) and adaptive immune response (T and B lymphocytes), effector cells (Vaishnava S et al., 2008) but also by the presence of tight junctions between the epithelial cells and physical barriers, such as keratin layers and mucous membranes.

The epithelial barriers are not permeable to microorganisms, in fact it has been demonstrated that the translocation of microbes and their products are carried out under physiological conditions, and the epithelial permeability may increase in infections, inflammations, and immunodeficient states. However, the mechanisms by which the microbiota modulates and participates in maintenance of inflammation and systemic immune diseases are not clear, although bacterial products and/or host factors produced response to them, and they can spread to distant and mediate effects of intestinal microbiota systemic immunity (Venkates M et al., 2014)

Round et al (Round JL et al., 2009) describe a healthy microbiota as a balanced composition of bacteria symbionts, commensals and pathobionts.

This condition is definited as eubiosis and it is responsible for many functions, such as the production of vitamin, hormonal activity, immunity and detoxification processes.

In conditions of dysbiosis there is an imbalance in the composition of the bacterial flora, resulting in a reduction in both the number of symbiotic and / or an increase in the number of pathobionts (Fig. 4); this condition can lead to disease state.

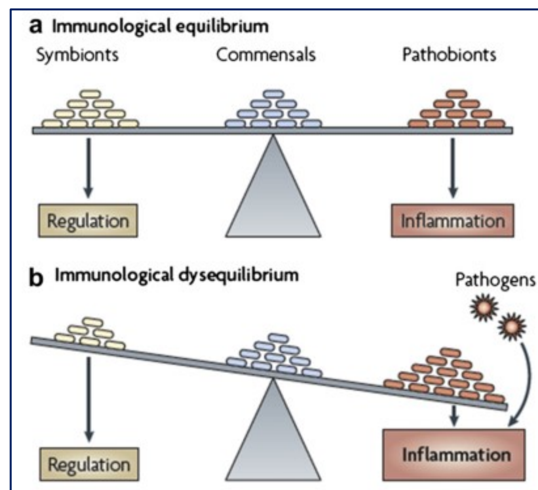


Fig. 4 The gut microbiota shapes intestinal immune responses during health and disease.

Dysbiosis causes a release of toxic metabolic products, followed by swelling, intestinal pain and inflammation. In addition, intestinal dysbiosis should be considered as a possible cause, or a contributing factor in patients who have for example autoimmune disorders, breast and colon cancer, in fact many chronic and degenerative diseases are considered today to be linked to abnormalities in the bacteria microflora ecology (Iannitti T and Palmieri B, 2010).

Many researches focused on the microbiota associated with tumors. In fact, growing evidence shows that the microbiota plays an important role in the cause of sporadic CRC; through metagenomic and metatranscriptomic studies has been shown, for the fecal microbiota of patients with CRC and polyposis, a

microbiome characterized by bacterial species that colonize selectively on-tumor and off-tumor sites (Arthur JC et al 2012; Marchesi JR et al., 2015).

Intestinal bacteria can promote the onset and progression of CRC by chronic inflammation state, genotoxin biosynthesis that interferes with cell cycle regulation, toxic metabolite production, or heterocyclic amine activation of pro-diet carcinogenic compounds (Candela M et al.,2014).

Chronic inflammation induces mutations, inhibits apoptosis or stimulate angiogenesis and cell proliferation (Grivennikov SI et al., 2010).

Dysbiosis determines an increased permeability of the mucosa, bacterial translocation, and the activation of the immune system resulting in an increased release of pro-inflammatory such as IL-12, IL-23, TNF-α, and INFγ, with subsequent activation of adaptive immune system cells, and various inflammatory mediators (Keku TO et al., 2015). This inflammatory causes the activation of transcription factors of NF-κB and STAT3 patways, (Hooper C et al., 2014; Tian J et al., 2003) and the generation of reactive oxygen and nitrogen species, driving oxidative stress, DNA damage, aberrant proliferation, and, finally, development of colorectal adenomas and cancer (Fig.5).

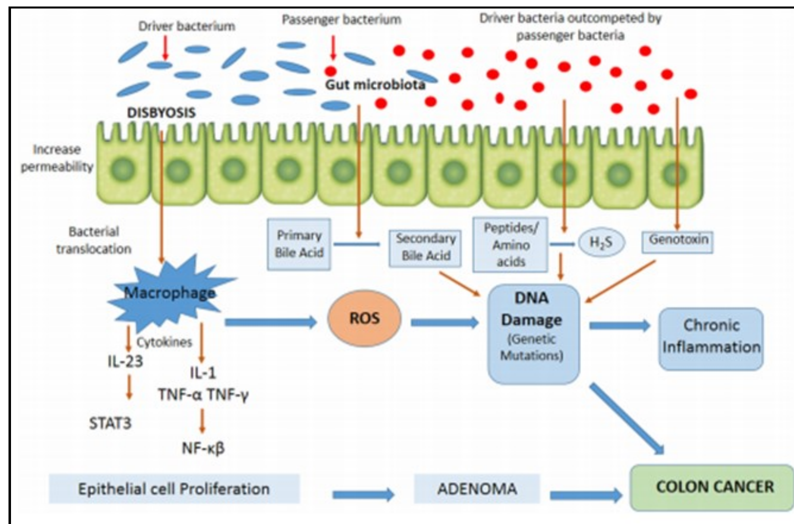


Fig. 5 Mechanisms possibly involved in microbial colorectal cancer promotion and the dynamic

Therefore colon cancer is a complex and multifactorial malignancy that involves genetic and environmental factors. In this context, there is a strong relationship between colon cancer, diet and intestinal microflora. Some species of bacteria in the colon produce harmful substances that appear to be related to cancer. Genotoxic bacterial metabolites can lead to mutations by binding specific cell surface receptors and affecting intracellular signal transduction. It was thought that probiotics can modulate several important intestinal functions potentially associated with the development of colon cancer in order to prevent the growth of harmful organisms, producing anti-cancer substances and promoting the balance between beneficial and harmful bacteria (Iannitti T and Palmieri B, 2010).

Moreover, the informations collected lead to the conclusion that different microbiota compositions may have different effects in immune signaling and that this can contribute to a chronic pro-inflammatory stimulus in the carcinogenesis of colorectal cancer.

Studies on the differences or alterations of intestinal flora have focused on the pathogenesis of CRC and these differences may be useful for screening (Schwabe and Jobin, 2013; Sears and Pardoll, 2011; Tjalsma et al., 2012). Comparisons of the fecal microbiota have been reported by four studies, totaling 176 CRC cases and 241 controls (Ahn et al., 2013; Zeller et al., 2014).

These studies reported that butyrate-producing bacteria were significantly less abundant in feces from CRC cases compared to controls, although the microbiota composition varied and three larger studies reported that CRC cases had significantly higher carriage or abundance of potentially pathogenic Fusobacteria and Proteobacteria. (Ahn et al., 2013; Wang et al., 2012; Zeller et al., 2014; J.J. Goedert et al., 2015).

Xuan et al investigated the potential role of microbiota in breast cancer by next-generation sequencing using breast tumor tissue and paired normal adjacent tissue from the same patient. They found that there is a difference of bacteria between normal and tumor tissue, particularly Methylobacterium radiotolerans is more present in tumor, while Sphingomonas yanoikuyae is more abundant in normal tissue; these two bacterial species were inversely correlated indicating that dysbiosis is associated with breast cancer. Furthermore, the total bacterial DNA was reduced in tumor versus normal breast tissue as determined by quantitative PCR; bacterial DNA correlated inversely with advanced disease, and this finding could have broad implications in diagnosis and staging of breast cancer. These data indicate that microbial DNA is present in breast and that bacteria or their components may influence the local immune microenvironment (Xuan C, et al., 2014).

5. MICROBIOTA AND DIET

Increasingly, the gut microbiome is implicated in the etiology of cancer, not only as an infectious agent, but also by altering exposure to dietary compounds that influence disease risk.

The relationship between diet, gut microbiota and cell molecular responses is well known; however, only recently the new technology made possible to identify strains of microorganisms harbored in the gastrointestinal tract that may increase susceptibility to disease, through changes in the gut microbiome resulting in metabolism alterations (Ibanez C, et al 2012; Riscuta G and Dumitrescu RG, 2012).

The composition and metabolism of the gut microbiota is influenced by diet and it can also modify dietary exposures in ways that are beneficial or harmful to the host.

The colonic bacteria metabolize macronutrients by different metabolic pathways; microbial metabolites can influence epigenetics by altering the pool of compounds used for modification or by directly inhibiting enzymes involved in epigenetic pathways. Colonic epithelium is exposed to these metabolites, although some of them are also found in systemic circulation.

Several studies have shown that there are significant geographic and seasonal variations in the gut microbiome. However, these differences were also associated with a difference in dietary patterns.

Thus, the diet is one the most important determinant in shaping composition, diversity and richness of intestinal

microbiota; a diet rich in fruits, vegetables and fibers is associated with a higher richness and diversity of the gut microbiota composition. Individuals that consume this kind of diet have a higher abundance of insoluble carbohydrate metabolizing organisms of the Firmicutes phylum.

Fruit, vegetables and other functional foods are active resources of bioactive compounds with various biological activities such as antibacterial, anti-oxidant, anti-inflammatory, anti-coagulant, anti-viral and apoptotic activity.

Several studies have demonstrated a significant shift in the gut microbiota upon the use of this foods supplement.

Fecal microbiota of healthy adults have substantial stability over time, but few studies have examined temporal changes in the whole gut microbial community in response to dietary change little. The influence of some dietary components can, however, be seen in carefully controlled human dietary studies. There is evidence that dietary supplementation with prebiotics can promote specific groups of bacteria (Walker AW et al., 2011).

Diet affects the composition of feces, in fact her components, the products of their digestion and metabolism are a source of mutagenic compounds that can cause neoplastic changes (Venturi Met al., 1997).

Diet also plays a vital role in maintaining the health and the dietary components directly interact with molecules produced by the microbiota. Thus, studying the relationship between the human gut microbiota, the epigenome and dietary components may offer new therapeutic options for treating and prevention diseases.

In recent years, the interest of researchers has concentrated in the aqueous phase of human feces (FW) to study the relationship with components of the diet, because FW interacts more readily with the epithelium of the colon compared to the solid phase and it reflects the luminal content of risk and protective factors (Woods JA et al., 2002; De Kok TMCM et al., 2000).

Therefore, several studies have shown that FW components are more effective altering growth conditions of colonocytes than the solid component (Rafter JJ et al., 1987; Lapre JA and Van der Meer R 1992). In fact, fecal water is a bioactive product highly variable able to cause a variety of cellular effects, such as DNA damage and cytotoxicity, this bioactivity can be altered through the diet (Bruce WR et al., 1977).

If the fecal water components are influenced by diet and possibly other unknown factors, a plausible hypothesis will be that biochemistry of the fecal water may differ between healthy individuals and patients.

Erba et al carried out a study on a group of healthy individuals and they showed that fecal water of these subjects, has low or moderate genotoxicity. The comparison of their results with those reported by other research groups shows a link between diet and FW activity, hypothesizing a correlation between diet and colon cancer mortality, also. The variability of the genotoxicity data inter-intra individual is due, presumably, to differences in diet and in the composition of the intestinal microbiota (Erba D et al., 2014).

In vitro studies using colon cancer cell lines show that fecal water components can influence different cellular activities important

for tumorigenesis such as genotoxicity, proliferation, apoptosis and cell signaling pathways (Glinghammar B and Rafter J, 2001).
Scientific researches on HT29 cell lines have shown that some FW components can able to modulate the activity of cyclooxygenase 2 (COX-2), and, in particular, a decrease of COX2 protein levels has been observed probably due to the presence of COX2 inhibitors in fecal water. These results were interesting because COX2 is involved in the carcinogenesis and inflammation processes, for this, FW could be seen as an important target for colon cancer prevention and treatment (Karlsson PC et al., 2005).
Literature suggests that protective components of the diet reflect on fecal water activity reducing its toxicity. The cytotoxicity and/or genotoxicity of fecal water is a potential biomarkers useful for studying the exposure bowel to carcinogenic agents and to evaluate how dietary habits can modify the intestinal environment (Pearson JR et al., 2009).
An increasing number of scientific evidence support the hypothesis that some foods and dietary components have beneficial, physiological and psychological effects.
Research is now focused on identifying biologically active food components; it was discovered, in fact, that many traditional foods possess compounds that show pharmacologic properties for the prevention and treatment of several disease.
In the light of these scientific evidence, between different functional foods we focused our study on *Lycium barbarum* or Goji berries, powerful "anti-aging" natural, also called "fruit of longevity"; it has stimulated great interest for its activities include antioxidant properties, antiaging effects, increased metabolism and antitumor activity (Jin M et al., 2013).

6. 2D MODEL CELL

6.1 Caco-2 cells as a model in vitro study on absorption intestinal

Emerging literature suggests that many dietary components can directly or indirectly regulate inflammatory response in the bowel by modulating the intestinal barrier functions. Furthermore, several dietary compounds have been shown as anti-cancer therapeutic and anti-oxidant agents. (Gupta SC et al., 2010).
The intestinal epithelium contains a heterogeneous population of cells such as enterocytes, Paneth cells, M cells, cells to clump and cells cup (Madara and Trier J, 1986).
In1974 Jorgen Fogh used a Caco-2 cell line model and in a few years it became the in vitro model of cell uptake accepted for studying drugs permeability. For compounds administered orally, the permeability through the monolayer of Caco-2 showed a good correlation with the in vivo absorption in humans.
This Caco-2 cell model allow to investigate main mechanisms of drug transport, absorption mediated by carriers and efflux mechanisms. This in vitro model of absorption is accessible and highly reproducible and it is used also for conduct toxicity analysis, as well as in the field of agribusiness (Shah P et al., 2006).
To better mimic the intestinal conditions in vivo, cells Caco-2 are grown on permeable insert which allows the exchange of ions and nutrients through the two sides of cell monolayer, called the apical and the basolateral side, in order to ensure an environment rich in nutrients for the incubation period. As these states allow good

morphological differentiation and functional, they have been proposed and widely used as a physiological model of the intestinal transport and toxicity studies.

However, conventional in vitro monolayer cell cultures that are frequently used for cell biology studies or for drug development not always mimic the cellular environment observed in vivo.

7. RATIONALE

Recent scientific evidences have highlighted the link between changes in the microbiota composition and chronic degenerative diseases susceptibility related to dietary modification because the microbiota plays an important role in the fermentation of food residues, in modulating gut immune function and protection against pathogens.

The aim of the study was to evaluate the beneficial and anti-oxidants effects of Goji berries on the intestinal bacterial composition through a preliminary study in humans.

In vitro cellular models using tumor lines were set up to test the chemo- preventive effects of this functional food measuring the change in the fecal water cytotoxicity in response to diet integration of berries of Goji.

Finally, 2D in vitro model performed in order to confirm the decrease of cytotoxic effects of fecal water after goji integration and to test its anti-inflammatory effects.

8. MATERIALS AND METHODS

8.1 Normal Subjects enrollment to evaluated L. barbarum effects on the fecal water and microbiota

The study was carried out with five volunteers (3 male and 2 female).

All subjects were healthy omnivores, nonsmokers with no history of gastro-intestinal disease or dietary supplements and antibiotic use in the previous 3 months; they were asked to provide complete fecal samples (the total feces of one passage).

The study was conducted with the informed consent of all participants.

8.2 Sample collection

Each volunteer collected one sample of stool at the enrollment and a second sample after 2 weeks. Volunteers were instructed to collect the first stool passed in the morning in disposable bedpans and to keep the sample refrigerated; the stool samples were transported to the laboratory within few hours, where aliquots of stool were immediately divided for microbiological assessment and for preparation of fecal water and stored at -80 °C.

8.3 Fecal water

Fecal water samples were prepared according to a well-established procedure designed to provide the free water fraction of stool to which the colonic epithelium is exposed in vivo. (Venturi et al., 1997). Samples were homogenized in a stomacher for 2 min and centrifuged at 50 000 g for 2 h at 20°C. The supernatant (fecal water) was carefully decanted into 1.5 ml Eppendorf tubes. All fecal water extracts were sterile filtered (Millipore, 0.45 µm and 0.20 µm) and stored at - 80°C until analysis. Sample weights and supernatant volumes were recorded.

8.4 Cell culture

The human colon adenocarcinoma cell line HT-29 and A375 (American Type Culture Collection) was cultured in monolayer in RPMI 1640 (supplemented with 10% fetal bovine serum (FBS), 2 mmol/L L-glutamine, and 1% penicillin/streptomycin), Caco-2 (American Type Culture Collection) was cultured in monolayer in EMEM (supplemented with 10% fetal bovine serum (FBS), 2 mmol/L L-glutamine, and 1% penicillin/streptomycin).
The cells were incubated at 37°C and 5% CO_2.

8.5 MTT Assay

Cell viability was evaluated by spectrophotometric analysis using MTT [3-(4,5-dimethylthiazolyl-2-yl)-2,5-diphenyl-tetrazolium bromide] (Sigma-Aldrich; St. Louis, MO).

A375 and HT29 cells were seeded in 96-well plates at 1.5x104cells/ml in a final volume of 200µl.
After 24 hours, each cell line was exposed to different concentrations (1%, 2%, 4%, 8%, 15%) of FW, obtained before and after administration of goji, for assessment of proliferation and cell viability by MTT assay at 24h, 48h and 72h of treatment. As negative controls were used the same cell lines with only medium.
MTT solution was added to each well at a final concentration of 0.5 mg/ml; after cells were further incubated at 37°C for 2-4 hours. Then, 100µl of isopropanol/HCL was added to each well to solubilize the formazan. Finally, absorbance A550 was measured using a microplate reader (Sunrise-TECAN).

8.6 2D Cell Culture

A monolayer intestinal differentiated cells was obtained using inserts MILLICELL-HA (polycarbonate membrane, 0,45 µm pore size, 30 mm diameter, Millipore). Insert were placed in 6-well plates. Caco-2 cells were seeded at 5 x 10^4 cells per cm^2 on the membrane insert with 1.75 ml of medium in the apical/luminal side (AP) and 3.25 ml of medium in the basolateral side (BL). Cells reached confluence within 5 days ; culture medium was changed three times a week. After 21 days from confluence, the EMEM was removed and the cells were washed three times with phosphate buffer saline (PBS).
The integrity of Caco-2 cell monolayer was evaluated by measuring the trans-epithelial electric resistance (TEER) using a

Millicell-ERS voltohmeter (Millipore Corp.,Bedford, MA). Only monolayers with TEER 300 Ω/ cm2 were utilized.
In experiments, the treatment was applied to differentiated cells grown in MILLICELL-HA inserts with integrity of monolayer.
For the treatments, cells were washed with PBS and each treatment was applied to cells:

- Control cells with medium alone
- TNFα (25ng/ml)
- Goji (1mg/ml)
- Goji + TNFα
- pre FW (8%)
- post FW (8%)

The membranes were recovered after 24h of treatment and cut in half; a part of the membrane was utilized for MTT assay and other part for the evaluation COX-2 gene expression by RetroTranscription-PCR (RT-PCR).

8.7 RetroTranscription-PCR (RT-PCR)

For the evaluation COX-2 gene expression total RNA was extracted using a PureLink RNA Mini Kit (Ambion Life technologies). The quantity of purified RNA was determined by measuring the absorption at 260nm using an ND-1000 spectrophotometer. Total RNA (1μg) was reversely transcribed using M-MLV reverse transcriptase (Invitrogen) to prepare a cDNA. PCR amplification

of cDNA was conducted using the following specific primers of COX-2 and PGK genes:

PGK1-FW TTAAAGGGAAGCGGGTCGTT

PGK1-RW CAGGCATGGGCACACCAT

COX2-FW CAGCACTTCACGCATCAGTT

COX2-RW AGACCAGGCACCAGACCAA

Take the amplified product was separated by 1% electrophoresis agarose gel.

8.8 Comet assay

To the Comet assay, Caco-2 cells were incubated with pre/post FW at concentration of 4%, 8% and 15%, in Eppendorf tubes for 30 min at 37°C.

Positive controls (100 μM H2O2) was included. Cells were centrifuged at 100 g for 3 min, the supernatant discarded and cells resuspended in 75 μl of 0.7% low melting point agarose (LMA; Gibco BRL, Life Technologies Inc., Gaithersburg, USA) made in PBS and maintained at 37°C for embedding on microscope slides. Cells were lysed (2.5 M NaCl, 100 mM Na2EDTA, 10 mM TRIS; containing 1% Triton X-100 and 10% dimethylsulphoxide) for 1 h at 4°C. The DNA was allowed to unwind in electrophoresis buffer (1 mM Na_2EDTA, 300 mM NaOH, pH 13.5) at 4°C for 20 min before electrophoresis for 20 min at 26V, 300mA (0.037 V/cm^2). After electrophoresis, slides were washed in neutralization buffer (0.4 M tris, pH 7.5) then stained and stored in a moist atmosphere at 4°C for no longer than 48 h before scoring. Slides were analyzed using a fluorescence microscope (Zeiss-Photomicroscope II).

8.9 Determination of bile acids by ELISA

The pre/post fecal water was collected and was evaluated using a sandwich ELISA method (Total Bile Acid ELISA Kit -Antibodies) in accordance with the manufacture's protocol.

8.10 DNA Bacterial Extraction

To prepare the sample for DNA extraction were collected from the entire 8gr stool sample, diluted 1: 4 with TE buffer (10mM Tris/Cl, 1mM EDTA, pH8) and homogenized by Stomacher (1 cycle 8 minutes). the samples were stored at -80 ° C.

Subsequently the sample was filtered thawed through the use of filters with pores of 5μm, to eliminate eukaryotic cells.

DNA was extracted from homogenized feces using NucleoSpin Tissue I of Macherey-Nagel following the instructions provided by the manufacturer. The final concentration of DNA was determined spectrophotometrically by Nanodrop1000.

8.11 Randomly amplified polymorphic DNA (RAPD) analysis

The discrimination among bacterial isolates at strain level was carried out by ramdom amplified polymorphic DNA (RAPD) analysis using random decamer primers (Table 1).

Table 1

Primer RAPD 2	5' AGTCAGCCAC 3'
Primer RAPD 5	5' CCGCAGCCAA 3'

Primer RAPD 6	5’ AGCAGCGTGG 3’
Primer RAPD 7	5’ TCGCCAGCCA 3’

Amplified products were separated by 2% agarose gel electrophoresis and visualized by staining with syber safe. Reproducibility was assessed using two different PCR thermocyclers (ThermalCyclerC1000 Biorad and MasterCycler epgradientS Eppendorf). No changes in banding profiles were observed.

The amplifications were carried out in a total volume of 50µl containing 50ng template DNA, in the reaction mixture following the next procedure (Table 2):

Table 2

Reagents	**Component volume (µl)**
Buffer	5 µl
dNTPs	5 µl
Taq Polimerasi	0,5 µl
Primer	2 µl
Template	1 µl
Water	37.5 µl

8.12 Amplified rDNA restriction analysis (ARDRA)

The differentiation among Lactobacillus, Streptococcus and Bifidobacterium genus was carried out by amplified rDNA restriction analysis (ARDRA).

The amplicons (910bp) used for restriction analysis were obtained by PCR with genus-specific primers PbiF1and PbiR2 and

they were digested with restriction endonucleases Alu I, Sau96I, Sau3AI, BamH1.

A 1004bp 16S rDNA gene fragment was amplified by PCR using the primers 16S1a and 16S1b, the amplicon was digested with restriction endonuclease HpaII.

A 1100bp 16S rDNA gene fragment was obtained by primers 16S Uni e E1115R; the PCR product was digested with restriction endonucleases AluI, HhaI, RsaI.

Table 3

16S1a	5’ GATTACATGCAAGTCGAACGA 3’
16S1b	5’ TTAACCCAACATCTCACGAC 3’
16S Uni	5’ AGAGTTTGATYMTGGCTCAG3’
E1115R	5’AGGGTTGCGCTCGTTG3’
PbiF1	5’ CCGGATAGCTCC3’
PbiR2	5’GACCATGCACCACCTGTGAA3’

ARDRA digests of each enzyme were separated in 2% agarose gel and visualized by staining with syber safe.

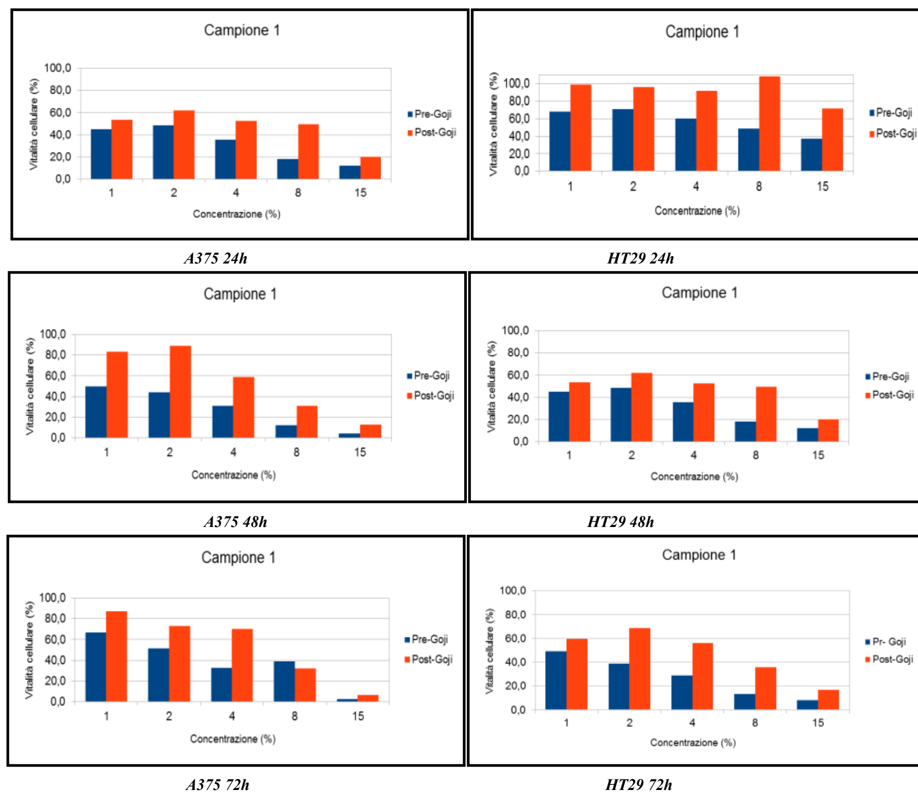
9. RESULTS

9.1 Decrease of cytotoxicity in Post goji Fw

The viability of the treated cells with FW, before and after diet integration with goji berries was measured by MTT assay.

The cell lines were incubated at 24h, 48h and 72h. Results obtained for both cell lines, A375 and HT29 are reported following.

The sample 1 shows a decrease in cell death, both in A375 and in the HT29, after FW post-goji treatment at 24h, 48h and 72h.

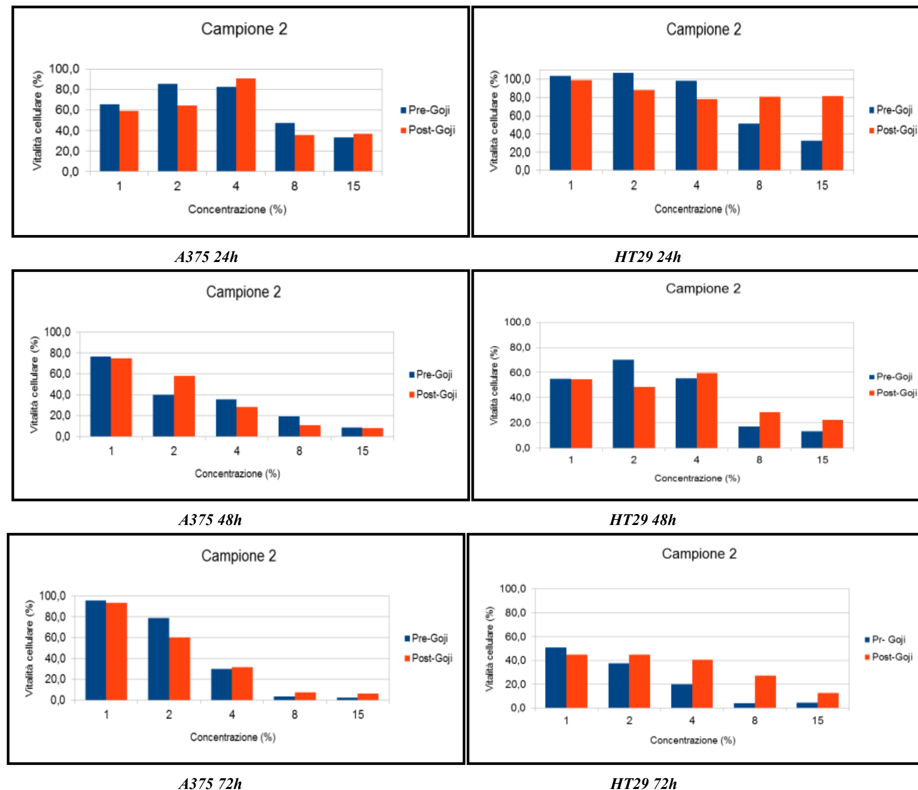


A375 24h *HT29 24h*

A375 48h *HT29 48h*

A375 72h *HT29 72h*

In sample 2, about the A375, there isn't a significant decrease in cell death between pre- and post-goji at various times of incubation; this is evident at higher concentrations post-goji FW in HT29.

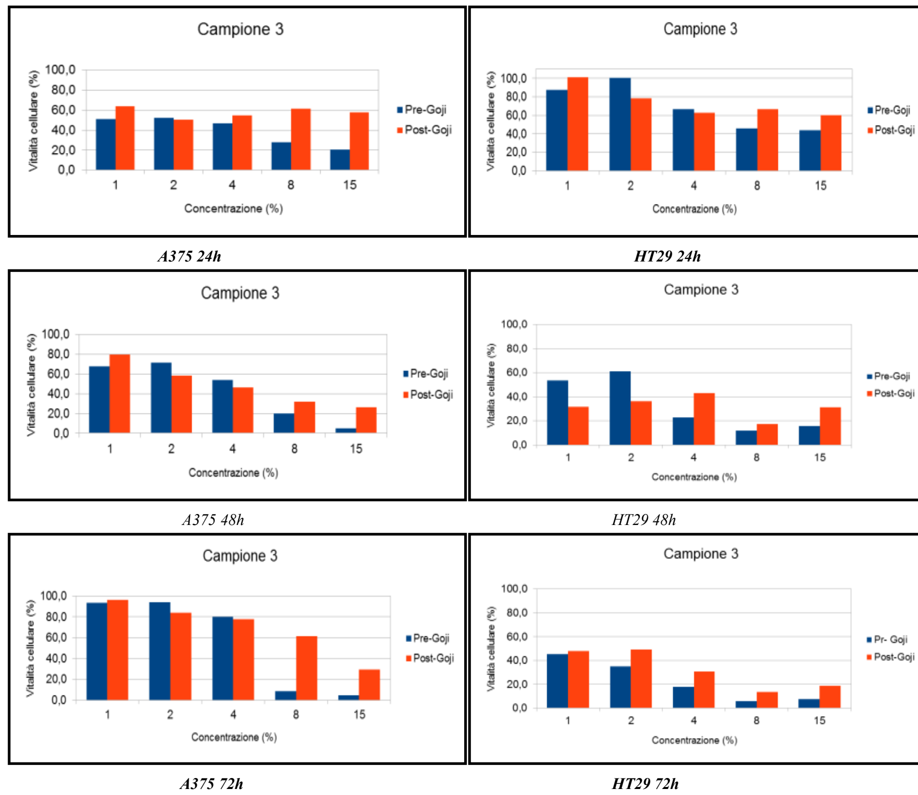


A375 24h HT29 24h

A375 48h HT29 48h

A375 72h HT29 72h

The sample 3 does not show, a significant variation of mortality at lower concentrations in A375treated with before and after goji FW, such variation is more pronounced at higher concentrations in post goji FW. In HT29 there is an increased vitality in post – goji FW treatment, compared to pre goji; it highlights the medium to high concentrations foe each incubation times.

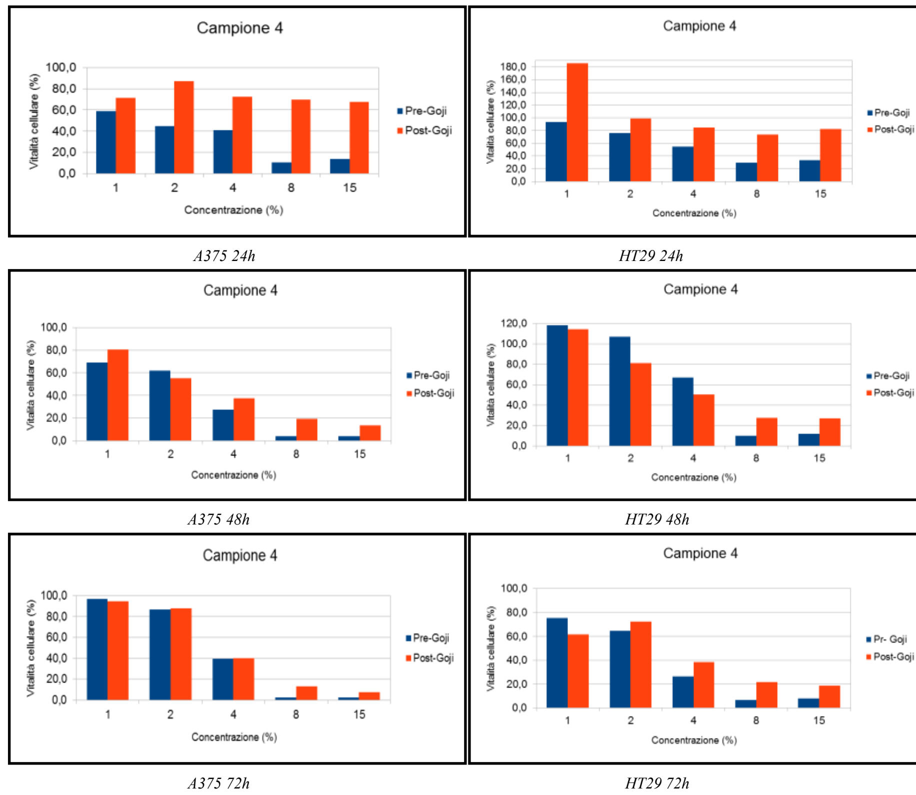


A375 24h *HT29 24h*

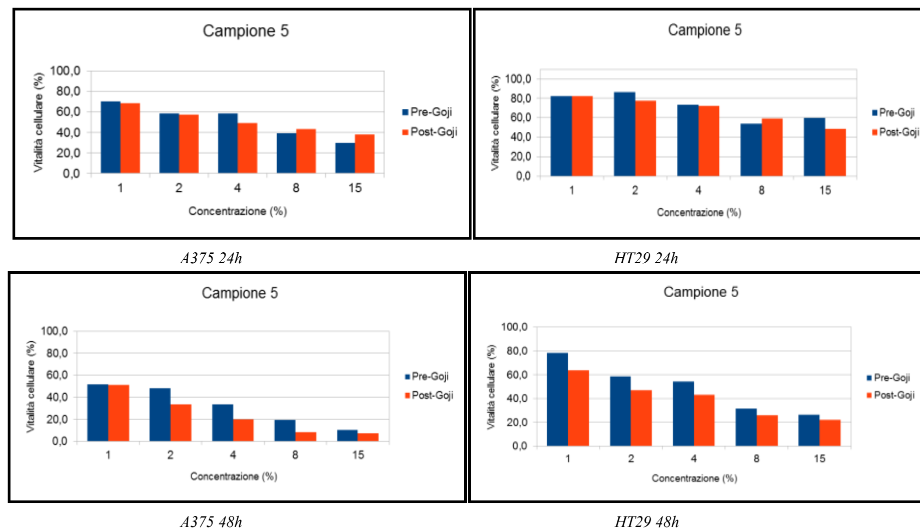
A375 48h *HT29 48h*

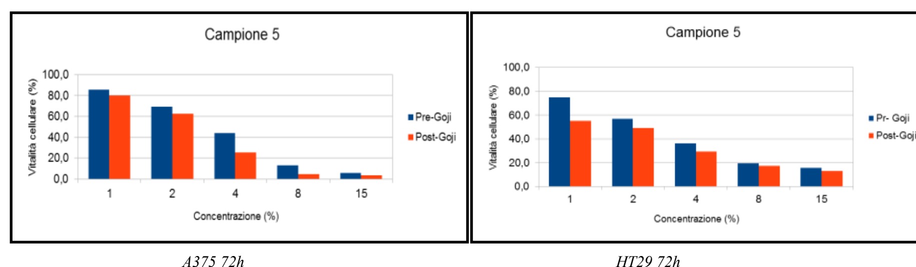
A375 72h *HT29 72h*

The sample 4 at 24h of incubation, in both cell lines, presents a cell death lower in the post-goji compared to pre-goji FW treatment. Instead, at 48h and 72h we observed a decreased mortality in the post-goji at concentrations of 8% and 15%.



The sample 5, in both cell lines, does not show significant changes in pre and post-goji FW treatment at 24h of incubation; at 48h and 72h mortality tends to increase.





A375 72h *HT29 72h*

Our results demonstrate that goji berries integration in the diet causes a decrease in fecal water cytotoxicity.

9.2 Anti-inflammatory activity of Lycium barbarum

For test viability, Caco-2 cell line was treated with TNFα, extract of goji berries and FW pre/post diet supplementation; the results obtained were compared to their CTRL.

The figure shows that vitality is lower in TNFα treatment and it increases when you add the goji berries extract; an increased viability is highlighted in treated cells with post-goji FW compared to pre-goji and the maximum value is showed in presence of goji berries extracted only.

As seen with monolayers culture cells, we find an increased vitality in post-FW treatment compared to pre-FW, although both vitality are higher than that recorded with TNFα, but it is still lower than that associated with goji berries extracted only.

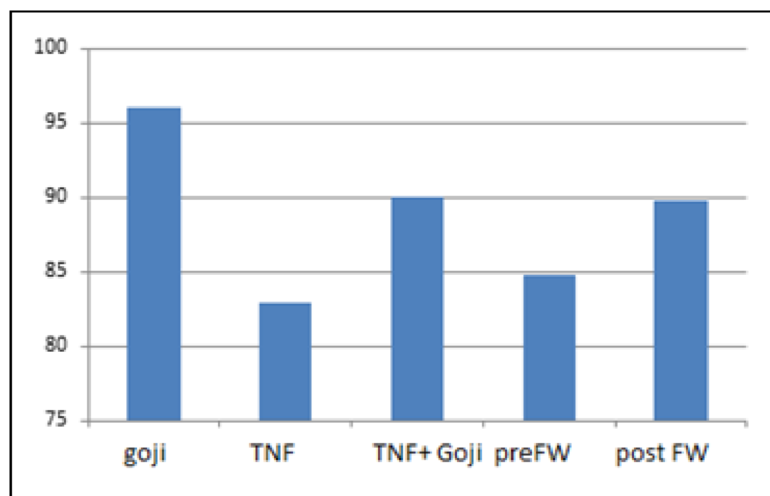


Fig.4 Caco2 2D model treatment: goji berries extract 1%; TNFα (25ng/ml); goji berries extract 1% and TNFα (25ng/ml); FW-pre e FW-post (8% sample 1).

Transepithelial electrical resistance (TEER) was determined in polarized Caco-2 cell monolayers using a Millicell-ERS Resistance System (Millipore), for the evaluation of the effects of TNFα and TNFα + goji. After a 10-min incubation, the initial TEER was determined (time 0) and incubated for a total period of 24h (Figure 5)

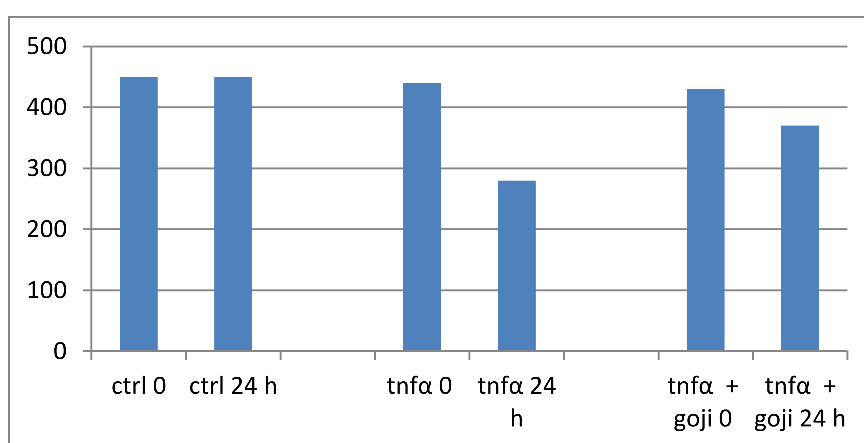


Fig.5 Effects of TNFα and TNFα +goji treatment on TEER in Caco2 cell

Results demonstrated that goji berries extract, in combination with TNFα, opposes of epithelial intestinal permeability induced by TNF .

To investigate the mechanisms involved in the treatment response, we carried out molecular analysis to understand the change in expression levels of COX2 gene.

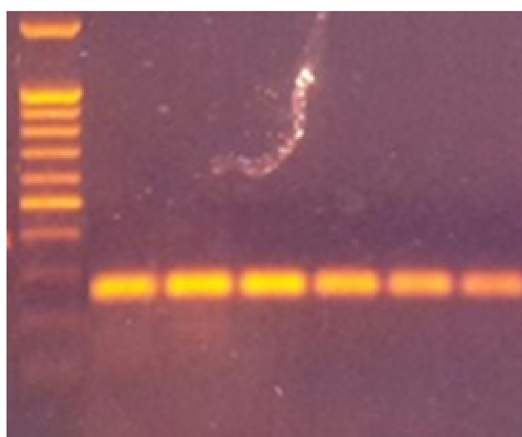


Fig. 6 Expression of COX2 gene

We have seen an increased COX2 expression in TNFα treatment compared to combination of TNFα and goji extract; it is higher in pre FW than post FW treatment (Fig 6).

In addition, COX2 shows a basal expression in goji extract treatment only.

The results show a modulation of gene expression that confirmed the data obtained by MTT assay.

9.3 Lower genotoxicity of post- goji fecal water

Genotoxicity of fecal water before and after goji berries integration was analyzed by using the Comet assay.

DNA damage induced by individual fecal water samples from the participants consuming goji berries is shows in Figure..

The results demonstrate an higher DNA damage in pre FW compared to post FW (Fig. 7).

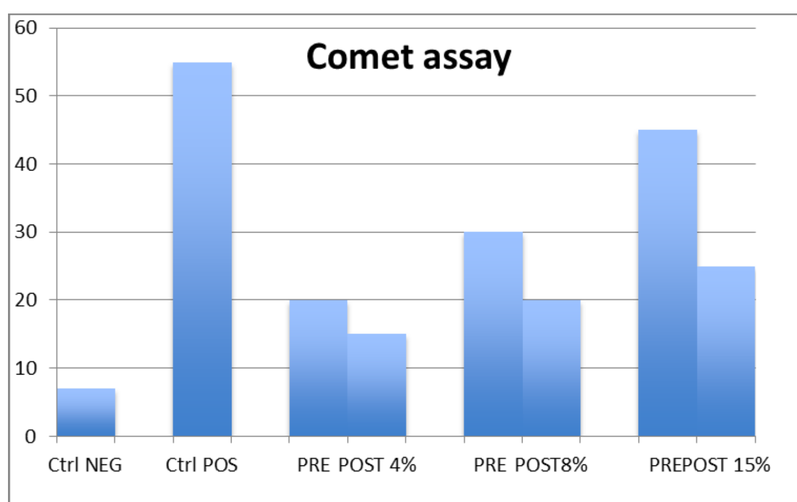


Fig. 7 Comet assay: genotoxicity of post- goji fecal water

The concentration of bile acid in fecal water both pre than post goji berries integration in the diet was measured by ELISA.

The results shows an higher presence of bile acid in the pre goji fecal water (Fig. 8). This is in accordance with the data obtained by MTT and Comet assay.

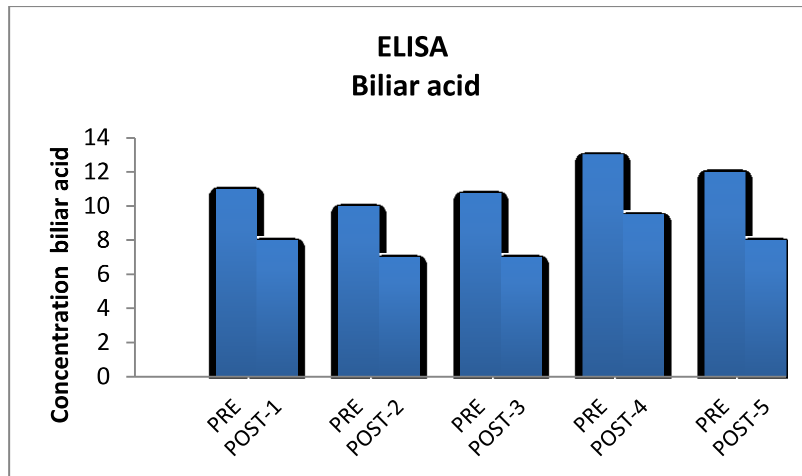


Fig. 8 ELISA assay: Concentration of bile acid

9.4 Variation of microbiota composition by RAPD and ARDRA

Randomly amplified polymorphic DNA (RAPD) technique allowed the discrimination of all bacteria by the performance of one PCR reaction.

The amplification products visualized by 2% agarose gel electrophoresis have generated profiles different for each individual; the strains formed many separate clusters without any potential for subspecies discrimination.

In fact the profiles obtained show a high inter and intra individual heterogeneity that does not allow us to identify the different bacterial profiles although each sample shows own profile.

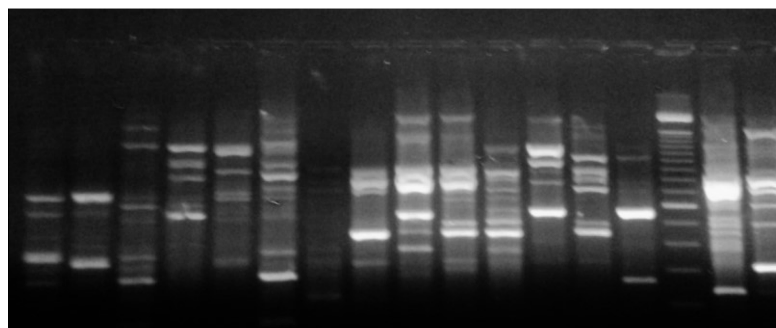


Fig. 9 RAPDs pattern

The ARDRA technique of the 16S rDNA gene allowed for the discrimination among three related genus (Lactobacillus, Streptococcus and Bifidobacterium) using restriction enzymes, since distinctive profiles were obtained for each genus.

A 1100 bp PCR product obtained with 16S primers Uni and E1115R were digested simultaneously with the enzymes AluI, HhaI, RsaI.

The results show different profiles among subjects both before and after goji berries integration.

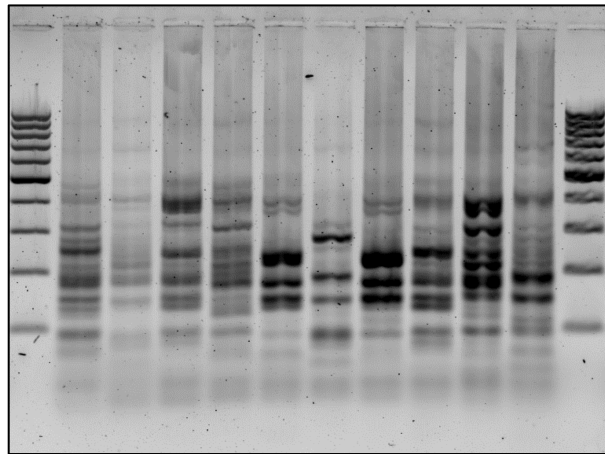


Fig.10 Enzymatic digestion with AluI, HhaI, RsaI; samples 1pre, 1post, 2pre, 2post, 3pre, 3post, 4pre, 4post, 5pre, 5post.

This does not allow us to distinguish and to attribute specific profiles among subjects in pre and post condition.

The amplicons (914 bp) obtained by PCR with specific primers PbiR2/PbiF1 for Bifidus spp., were digested with enzymes Alu I, Sau96I, Sau3AI, BamH1.

The digestion patterns obtained for each enzyme have allowed us to see the massive presence of Bifidobacterium spp. genus.

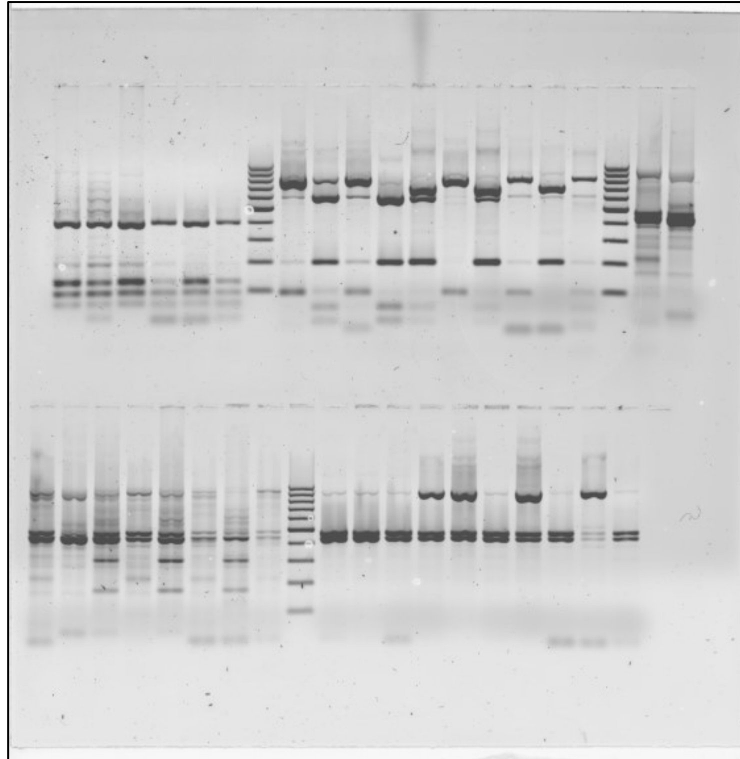


Fig.11 Enzymatic digestion with: Alu I, Sau96I, Sau3AI, BamH1; samples 1pre, 1post, 2pre, 2post, 3pre, 3post, 4pre, 4post, 5pre, 5post.

A 1004 bp PCR product obtained with primers 16S1a and 16S1b, were then digested with the enzyme HpaII.

Again, we obtained profiles consist of different patterns resulting from HpaII on 3 genus taken into account, such as Bifidobacterium, Lactobacilli and Streptococci.

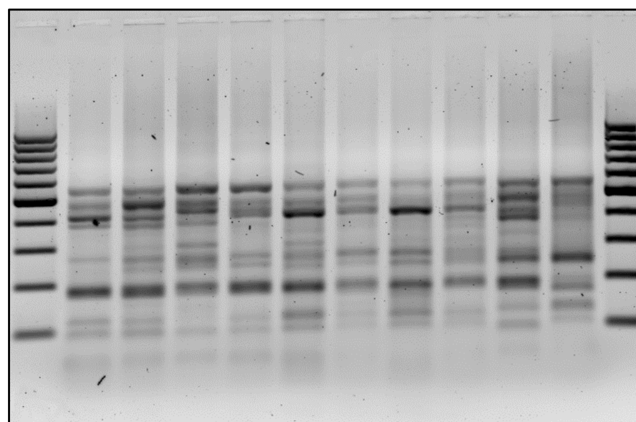


Fig. 12 Enzymatic digestion with HpAII: samples 1pre, 1post, 2pre, 2post, 3pre, 3post, 4pre, 4post, 5pre, 5post

Even in this case, we noticed a difference between different obtained profiles, without being able to define particularly changes.

10. DISCUSSION

The human organism has a complex ecosystem microbial involved in the development of metabolic and inflammatory diseases and cancer. In this direction, important is understand the molecular mechanisms of the host-organism relationships and between different microbial species.

Several studies are evaluating the possible relationships between intestinal flora alterations, diet and chronic diseases; the body's response to diet and its susceptibility modulation to various diseases, are central to understanding the role of the microbiota in preserving and improving human welfare.

Changes in diet affect the intestinal flora and, conversely, the microbiota may increase the production of mutagenic compounds that can cause pathological changes; therefore, the identification of microbial communities associated with carcinogenesis is of fundamental importance.

Numerous studies are aimed at understanding the impact of diet on the microbiota and on how the use of some functional foods can improve the intestinal environment, obtaining improved health status and/or reducing the risk disease.

Thus it is important identify foods that combine, in a synergistic way, nutritional and pharmacological aspects.

L. barbarum berries have an important role as main ingredient in natural remedies, because they show a wide variety of biological proprieties and active compounds with pharmacological

functions that could be used in the prevention and treatment of various chronic diseases.

Several studies have shown that the polysaccharide is responsible for biological activities of *L. barbarum*, it also have important biological activities, such as antioxidant, immunomodulatory, anti-degenerative, neuroprotective, anti-diabetic, hepato-protective and anti-cancer.

In this regard, some experimental models based on the use of the fecal water allow to study this interaction, because it interacts more easily with the colon epithelium compared to the solid phase; the fecal water reflects the luminal contents of risk and protective factors.

Cytotoxicity fecal water is a potential biomarker useful for studying the intestinal exposure to carcinogens and to evaluate how dietary habits can change the intestinal environment.

Based on scientific evidence, we evaluated the possible effects of goji berries on the fecal water activity.

For this purpose, we have set up an "in vitro" model using tumor cell lines, A375 and HT29, which were treated with FW before and after integration of goji berries for different times.

The results show that the integration of goji berries leads to a reduction of the post FW cytotoxicity in most of the samples, demonstrated by a decrease in mortality in both cell lines. However, HT29 compared to A375 appear to be less sensitive to fecal water toxicity.

The different behavior observed between the samples could be linked to different dietary habits, the would be appropriate that all participants have the same diet to try to minimize inter-individual variability.

To confirm this data obtained we set up a 2D model to create in vitro the intestinal conditions. In this context, we evaluated the inflammatory response following stimulation with TNFα.

Data demonstrated that goji berries decrease the inflammation.

Because fecal water is a bioactive toxic compound we also evaluated the genotoxicity to see how FW can induce DNA damage. As expected, we founded a higher genotoxicity in pre FW and, in addition we founded a higher concentration of bile acids, as reported in literature.

Given that the goji berries integration causes changes of intestinal environment we have seen its effects in the composition of the microbiota.

By molecular techniques we noticed a different bacterial profiles with a greater presence of Bifidus genus following goji berries integration, confirming an improved intestinal environment.

FW as mediating many diet effect could be a risk marker for colon cancer.

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